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An Evaluation of Avian Influenza Diagnostic Methods with Domestic Duck Specimens

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SUMMARY. Monitoring of poultry, including domestic ducks, for avian influenza (AI) virus has increased considerably in recent years. However, the current methods validated for the diagnosis and detection of AI virus infection in chickens and turkeys have not been evaluated for performance with samples collected from domestic ducks. In order to ensure that methods for the detection of AI virus or AI virus antibody will perform acceptably well with these specimens, samples collected from domestic ducks experimentally infected with a U.S. origin low pathogenicity AI virus, A/Avian/NY/31588-3/00 (H5N2), were evaluated. Oropharyngeal (OP) and cloacal swabs were collected at 1, 2, 3, 4, 5, 7, 10, 14, and 21 days postinoculation (PI) for virus detection by virus isolation, which was considered the reference method, and real-time RT-PCR. In addition, two commercial antigen immunoassays were used to test swab material collected 2–7 days PI. Virus isolation and real-time RT-PCR performed similarly; however, the antigen immunoassays only detected virus during the peak of shed, 2–4 days PI, and both kits detected virus in fewer than half of the samples that were positive by virus isolation. Cloacal swabs yielded more positives than OP swabs with all virus detection tests. To evaluate AI virus antibody detection serum was collected from the ducks at 7, 14, and 21 days PI and was tested by agar gel immunodiffusion (AGID) assay, a commercial blocking enzyme-linked immunosorbent assay (ELISA), and homologous hemagglutination inhibition (HI) assay, which was used as the reference method. Results for the ELISA and HI assay were almost identical with serum collected at 7 and 14 days PI; however, by 21 days PI 100% of the samples were positive by HI assay and only 65% were positive by ELISA. At all time points AGID detected antibody in substantially fewer samples than either ELISA or HI assay.

RESUMEN. Evaluación de los métodos diagnósticos para la influenza aviar con muestras de patos domésticos.

El muestreo para la influenza aviar en aves comerciales incluyendo patos domésticos se ha incrementado en los años recientes. Sin embargo, los métodos actuales validados para el diagnóstico y detección de la infección con el virus de la influenza en pollos y pavos no han sido evaluados en su desempeño con muestras recolectadas de patos domésticos. Para asegurar que los métodos para la detección del virus de influenza o de los anticuerpos contra este virus funcionan de manera aceptable con estos especimenes, se evaluaron muestras recolectadas de patos domésticos infectados experimentalmente con un virus de influenza de baja patogenicidad con origen en los Estados Unidos, A/Avian/NY/31588-3/00 (H5N2). Hisopos orofaríngeos y cloacales se recolectaron en los días 1, 2, 3, 4, 5, 7, 10, 14 y 21 después de la inoculación para detectar al virus de influenza mediante aislamiento viral, considerado el método de referencia y por transcripción reversa y reacción en cadena de la polimerasa (de la siglas en inglés RT-PCR) en tiempo real. Además, dos inmunoensayos comerciales para la detección de antígeno viral fueron utilizados para evaluar el material de hisopos recolectados de 2 a 7 días después de la inoculación. El aislamiento viral y la RT-PCR en tiempo real funcionaron de manera similar, sin embargo, los inmunoensayos para detectar antígeno únicamente detectaron al virus durante el periodo de mayor eliminación viral entre los 2 y 4 días después de la inoculación, ambos ensayos detectaron el virus en menos de la mitad de las muestras que fueron positivas por aislamiento viral. Se obtuvieron más resultados positivos con los hisopos cloacales en comparación con los hisopos orofaríngeos en todos los métodos de detección. Para evaluar la detección de anticuerpos contra el virus de la influenza aviar, muestras de suero recolectadas de los patos a los 7, 14 y 21 días después de la inoculación fueron evaluadas mediante inmunodifusión en gel de agar, mediante un ensayo comercial competitivo de inmunoabsorción ligado a enzimas (de las siglas en inglés ELISA) y la prueba homóloga de inhibición de la hemoaglutinación (de las siglas en inglés HI), la cual fue utilizada como método de referencia. Los resultados de los ensayos de ELISA y HI fueron casi idénticos con los sueros recolectados a los 7 y 14 días, sin embargo, por el día 21 después de la inoculación, 100% de las muestras fueron positivas por HI y solamente el 65% de las muestras fueron positivas por ELISA. La prueba de inmunodifusión en agar detectó anticuerpos sustancialmente en menos muestras en comparación con las pruebas de ELISA o HI en todos los muestreos realizados.

Key words: avian influenza virus, waterfowl, domestic ducks, avian influenza monitoring, virus detection, antibody detection

Abbreviations: AGID = agar gel immunodiffusion; AI = avian influenza; bELISA = blocking enzyme-linked immunosorbent assay; HA = hemagglutination; HI = hemagglutination inhibition; HPAIV = highly pathogenic avian influenza virus; LPAIV = low pathogenicity avian influenza virus; OP = oropharyngeal; PI = postinoculation; rRT-PCR = real-time reverse transcription polymerase chain reaction; VI = virus isolation

Monitoring and preslaughter testing of domestic poultry flocks for avian influenza (AI) virus has increased substantially due to the establishment of an AI virus control program in the National Poultry Improvement Plan and the increased concern about the potential introduction of Asian H5N1 highly pathogenic AI virus (HPAIV) into the United States. Until recently, most of the focus of AI virus testing has been on chickens and turkeys; however, with the important role of domestic ducks and other waterfowl in the continued circulation of Asian H5N1 HPAIV, the emergence of strains of AI virus that cause disease and mortality in ducks, and as public awareness of AI virus has increased, the need for rapid and cost effective methods for surveillance and monitoring of domestic ducks for both HPAIV and low pathogenicity AI virus (LPAIV) is needed.

Numerous rapid diagnostic and detection tests for AI virus have been available for use with specimens from chickens and turkeys

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(1,8,12) and it is expected that these tests should perform similarly well in ducks; however, the species tested can affect diagnostic test performance because of 1) differences in sample types due to species specific tissue tropism, 2) sample composition due to environmental and dietary differences, 3) differences in host immune response (e.g., antibody detection), and 4) host adaptation of the virus. Therefore it is necessary to evaluate AI virus detection tests which have been optimized for chickens and turkeys with specimens from domestic ducks. Here we report the evaluation of real-time RT-PCR (rRT-PCR) and two commercial immunoassays for virus detection using virus isolation as a reference standard, and we report the comparison of agar gel immunodiffusion (AGID) assay and commercial blocking enzyme-linked immunosorbent assay (bELISA) with homologous hemagglutination inhibition (HI) assay as a reference standard for antibody detection with specimens from ducks experimentally exposed to LPAIV.

MATERIALS AND METHODS

Virus. A LPAIV that is genetically representative of North American wild bird AI viruses, A/Avian/NY/31588-3/00 H5N2 (5,10), was selected for use in the experimental infections. Virus stocks were propagated and titrated in embryonating chicken eggs by standard procedures (12).

Experimental infection of ducks. Forty-eight 3-wk-old domestic ducks (*Anas platyrhynchos*) were obtained from a commercial hobby-bird hatchery (Privett Hatchery, Portales, NM). The ducks were separated into two groups. One group of eight ducks served as uninoculated controls and was housed in battery cages with *ad libitum* access to feed and water. The remaining group of 40 ducks was housed within HEPA filtered isolation units located in a BSL-3 enhanced facility. Birds were provided with *ad libitum* access to feed and water. The group of 40 ducks was inoculated with 10^6 EID_{50}/bird of A/Avian/NY/31588-3/00 H5N2 LPAIV by the intranasal route. All animal care procedures were conducted in accordance with institutional animal care and use guideline.

Cloacal and oropharyngeal (OP) swabs for virus isolation, antigen immunoassay, and rRT-PCR were collected from all ducks at 1, 2, 3, 4, 7, 10, 14, and 21 days postinoculation (PI). At 7, 14, and 21 days PI serum was collected from all ducks for HI assay, bELISA, and AGID assay. The birds were euthanatized in accordance with institutional animal and care use guidelines at 21 days PI.

Virus isolation. Virus isolation (VI) was attempted with all OP and cloacal swabs in embryonating chickens eggs in accordance with standard procedures (12) using three eggs per swab and 200 µl of swab material per egg. Prior to egg inoculation the swabs were treated with antibiotics at a final concentration of 2 µg/ml amphotericin B; 1000 U/ml penicillin G; and 100 µg/ml gentamicin for 1 hr at room temperature. Hemagglutination assay was used to evaluate virus replication in inoculated eggs and 30 random hemagglutination positive samples were confirmed to be positive for AI virus with a commercial immunoassay (BinaxNOW influenza A & B, Inverness Medical, Portland, ME).

RNA extraction. RNA was extracted from cloacal and OP swabs by adding 750 µl Trizol LS (Invitrogen, Inc., Carlsbad, CA) to 250 µl swab material. The swab material–Trizol LS mixture was mixed by vortexing and incubated at room temperature for 5 min, then 200 µl of chloroform was added. The material was mixed by vortexing, incubated at room temperature a minimum of 10 min and centrifuged for 15 min at 12,000 × *g*. Instead of precipitation with 2-propanol according to the manufacturer's instructions, the RNA extraction was completed by binding and eluting the RNA from the aqueous phase using the MagMAX 96 AI/ND Viral RNA isolation kit (Ambion, Inc. Austin, TX) in accordance with kit instructions using the KingFisher magnetic particle processor (Thermo Scientific, Waltham, MA).

Real-time RT-PCR. Quantitative rRT-PCR which targets the influenza M gene (9) was performed using the 7500 FAST Real-time PCR System, (Applied Biosystems, Foster City, CA) and the AgPath-ID OneStep RT-PCR kit (Ambion, Inc.) in accordance with the U.S. National Veterinary Services Laboratories protocol AVSOP1521.01. The standard curve for virus quantification was established with RNA extracted from dilutions of the same titrated stock of A/Avian/NY/31588-3/00 used to inoculate the ducks and was run in duplicate.

Commercial antigen immunoassays. Two commercial strip format AI virus antigen detection assays: BinaxNOW influenza A & B (Inverness Medical, Inc., Waltham, MA) (assay A) and Flu Detect (Synbiotics, Inc., San Diego, CA) (assay B), were used to evaluate OP swabs from 2–4 days PI and cloacal swabs from 2–7 days PI. Each kit was run in accordance with the manufacturer's instructions.

Hemagglutination inhibition assay. HI assay was performed in accordance with standard procedures (13). Briefly, twofold serial dilutions of 25 µl of serum were made in 25 µl of phosphate-buffered saline. Diluted sera were incubated for 30 min at room temperature with 4HAU/25 µl of A/Avian/NY/31588-3/00 virus, then 50 µl of 0.1% chicken red blood cells were added. The test was evaluated after 30 min of incubation at room temperature. Titers were calculated as the reciprocal of the last HI positive serum dilution and samples with HI titers of 8 or below were considered negative.

AGID assay. AGID was performed using standard procedures (13). Reference antigen and antibody were obtained from the National Veterinary Services Laboratories (USDA, APHIS, Ames, IA). The assay plates were read at both 24 and 48 hr of incubation.

ELISA. The multiS-screen commercial ELISA AI virus antibody test kit (IDEXX laboratories, Westbrook, ME) was used and was evaluated according to the manufacturer's instructions. This ELISA uses a bELISA format, therefore is expected to work with sera from any species.

Statistical methods. The Cohen Kappa statistic (2) was used to evaluate the agreement between virus isolation and rRT-PCR, between virus isolation and each immunoassay, between HI assay and bELISA and between HI and AGID assay.

RESULTS

Virus detection. Virus isolation was attempted with all cloacal and OP swabs. No virus was detected in specimens from the uninoculated control ducks at any time by any method.

Virus could be detected by VI in OP swabs starting at day 1 PI and continued through day 14 PI (Fig. 1); all the ducks were positive 2 days PI. Cloacal swabs were positive for virus isolation at all time points; the most ducks (93.7%) were positive at 2 days PI (Fig. 2).

Oropharyngeal swabs were tested from 2 to 4 days PI and cloacal swabs were tested from 2 to 7 days PI with each of two commercial immunoassay kits. Virus was detected in only one OP swab with immunoassay B at 2 days PI, no OP swabs were positive with immunoassay A at any time (Fig. 1). With cloacal swabs, immunoassay A detected virus at days 2 and 3 PI and immunoassay B detected virus from 2 to 4 days PI (Fig. 2). The comparative results for the commercial immunoassays and VI are shown in Table 1. Overall, immunoassay A detected 18.8% and immunoassay B detected 45.4% of the total samples that were positive for VI. Agreement between each immunoassay and VI was very poor as evaluated with the kappa statistic, which was <1 for both immunoassays.

Real-time RT-PCR detected virus in OP swabs from 1 through 7 days PI (Fig. 1) and in cloacal swabs from 1 through 10 days PI (Fig. 2). Overall rRT-PCR detected virus in 116 of 135 (85.9%) of the OP swab samples that were positive for VI (Table 1) and 113 of 129 (87.6%) of the VI positive cloacal swabs (Table 1). The agreement between VI and rRT-PCR was evaluated with the kappa statistic, which was 0.65 for OP swabs and 0.72 for cloacal swabs.

The rRT-PCR assay was run with a standard curve to determine the amount of virus in positive samples. The mean titer of virus detected in OP swabs peaked 2 days PI with a mean titer of $10^{3.1}$ EID_{50}/ml. Virus

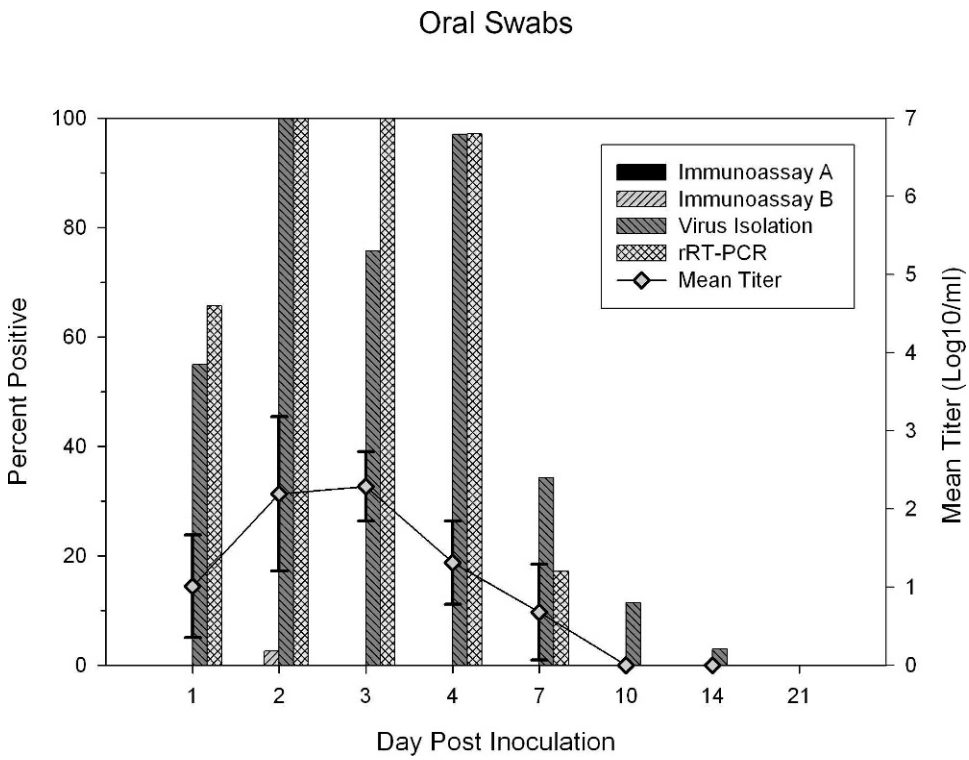

Fig. 1. Percentage of OP swabs from experimentally inoculated ducks that were positive for AI virus detection by assay and day PI. Error bars for virus titer denote the standard deviation.

titers in cloacal swabs were higher than OP swabs at all sample times with a peak at 2 days PI (Fig. 2) with a mean titer of $10^{5.4}$ EID_{50}/ml.

Antibody detection. Antibody was detected in serum samples by AGID, HI assay, and bELISA at 7, 14, and 21 days PI (Fig. 3). Overall HI and bELISA had much better agreement than either test did with AGID: 75 of 97 samples (77.3%) were positive by both tests, 17 samples 17.5% were only positive by HI, five samples (5.1%) were only positive by bELISA, and no samples were negative

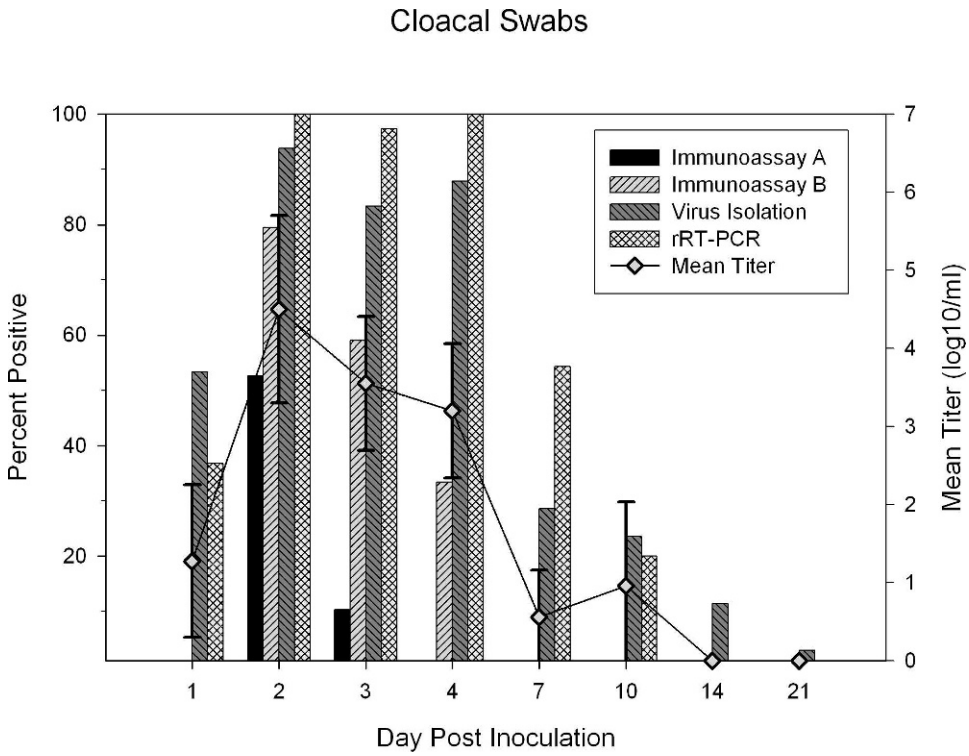

Fig. 2. Percentage of cloacal swabs from experimentally inoculated ducks that were positive for AI virus detection by assay and day PI. Error bars for virus titer denote the standard deviation.

Table 1. Avian influenza virus isolation results for oral and cloacal swabs from experimentally inoculated ducks compared with commercial immunoassays A and B and rRT-PCR.

		Immunoassay A cloacal swabs (n = 132)		Immunoassay B cloacal swabs (n = 133)		rRT-PCR oral swabs (n = 284)		rRT-PCR cloacal swabs (n = 282)	
		Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Virus isolation	Pos.	18 (13.6%)	78 (59.1%)	44 (33.1%)	53 (39.8%)	116 (40.8%)	19 (6.7%)	113 (40.1%)	16 (5.7%)
	Neg.	2 (1.5%)	34 (25.7%)	8 (6.0%)	28 (21.0%)	21 (7.4%)	128 (45.1%)	33 (11.7%)	120 (42.5%)

with both assays. At all time points AGID detected antibody in fewer samples than either bELISA or HI (Fig. 3). There was one sample on days 14 and 21 each that was positive by AGID, but not HI assay. At 7, 14, and 21 days PI, 91.4%, 94.2%, and 100%, respectively, of the serum samples were positive by HI. By bELISA, 91.0%, 91.0%, and 65% of the samples were positive at 7, 14, and 21 days, respectively. Antibody was detected by AGID in 48.6%, 22.8%, and 28.1% of the samples at 7, 14, and 21 days PI, respectively (Fig. 3). Agreement between HI assay and either bELISA or AGID assay was poor based on kappa statistics of <1 for each when compared to HI assay.

DISCUSSION

Three methods of AI virus detection were compared with specimens from domestic ducks experimentally inoculated with a LPAIV. Both VI and rRT-PCR performed similarly and it appears that either would be acceptable for virus detection as the correlation was good statistically with a kappa value of 0.65 and 0.72 for OP and cloacal swabs respectively, which is in the range accepted to describe substantial agreement (0.61–0.80). Virus isolation did offer a longer window of virus detection after infection, which suggests better sensitivity, but that must be considered against the practical advantages of rRT-PCR, which are lower cost and faster results.

Antigen detection assays are cheap and easy to use, can be run on-site, and provide results faster than other methods. Therefore, they are frequently used in the field; however, they have limited sensitivity. In this study the antigen immunoassays only detected the virus for a short time at the peak of virus shed between days 2 and 4 PI and most of the positive samples were cloacal swabs.

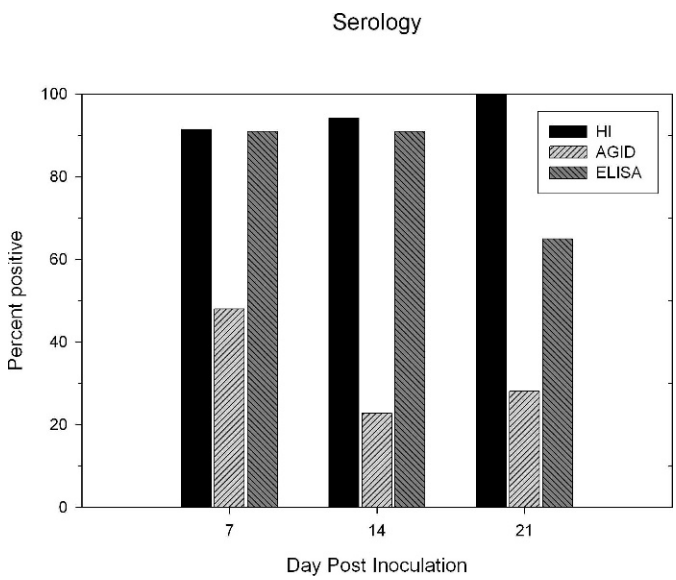

Fig. 3. Percentage of serum samples from experimentally inoculated ducks positive for AI virus antibody by detection assay and day PI.

Detection seemed to correlate with titer (Fig. 1, 2), which is consistent with the sensitivity reported by the manufacturers. Because of their limited sensitivity, it is recommended to target sick or dead birds when using the immunoassays with chicken and turkey specimens; however, since ducks do not typically show clinical signs of AI virus infection this approach may not be as beneficial, therefore it may be necessary to use only the more sensitive methods for detection of LPAIV.

Three methods, HI, bELISA, and AGID, which are well-established methods for AI virus antibody detection in chicken and turkey serum, were compared for AI virus antibody detection in duck serum. AGID assay performed poorly, which was not unexpected since the inconsistent reactivity of AGID with duck serum has been previously reported (7), but this is the first reported comparison of AGID with a reference method for duck serum. HI assay appeared to be the most sensitive because 18.4% of the samples overall were positive by HI alone, suggesting that bELISA and AGID were missing positive samples. Unfortunately, HI assay is not practical as a routine diagnostic test unless only certain subtypes are targeted; even then sensitivity will vary depending on the relationship between a field strain and the strain used in the HI assay. In this report the sensitivity was particularly good since the homologous strain could be used. One unexpected serological result was that at 21 days PI 11 samples were HI positive, but negative by bELISA, the reason for this is unclear since the HI titers at 21 days PI were similar to 7 and 14 days PI, thus ruling out a difference in sensitivity. Although bELISA was somewhat less sensitive than HI assay, it is more practical, especially on a flock basis, and it had substantially better sensitivity than AGID.

The peak of virus shed observed here was 1–4 days PI. However, with natural infection not all birds are infected at the same time, therefore the detection window should be longer on a flock basis. Additionally, and in accordance with previous studies that have demonstrated the intestinal tropism of LPAIV in waterfowl (3), cloacal swabs were a better sample type than oral swabs with this isolate, which was a wild bird isolate that was not adapted to poultry.

Although detection of the Asian H5N1 HPAIV in duck-origin specimens was not addressed by this study, the optimal paradigm for H5N1 monitoring would likely not be the same as for LPAIV. Experimental testing for the Asian H5N1 from numerous reports has shown that the Asian H5N1 HPAIV is shed at higher titers from the oro-pharynx/trachea than from the cloaca (4,6,14) in contrast to LPAIV and other HPAIV strains. Additionally, because some strains of the Asian H5N1 HPAIVs since 2002 may cause clinical disease in some species of ducks (11), targeting sick birds would be possible, which may mean that the antigen detection immunoassays would have more utility. Importantly, without previous knowledge of the circulating virus, differences in tissue tropism of different virus isolates make the use of a single sample type, OP or cloacal, impractical if high sensitivity in all cases is the goal.

This report addresses detection of LPAIV in domestic ducks with common diagnostic methods for AI virus in poultry. The virus detection methods performed similarly with duck specimens as they

do with chicken and turkey origin samples and demonstrated that they can be applied for use with duck specimens with a reasonable expectation of sensitivity and specificity. However, there were differences with antibody detection since one of the most common tests for AI virus antibody for chickens and turkeys, AGID, did not perform well with duck sera. Therefore alternative methods such as the bELISA should be considered. Along with economic and practical considerations, the performance of these tests needs to be taken into account when developing AI virus monitoring programs for domestic ducks.

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